Potential Application of D-Optimum Designs in the Efficient Investigation of Cytochrome P450 Inhibition Kinetic Models

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Abbreviations used: P450 or CYP, cytochrome P450; CPR,NADPH, Nicotinamide adenine dinucleotide phosphate; High performance liquid chromatography, HPLC; LC/MS/MS, liquid chromatography-tandem mass spectrometry.
Abstract.

Correctly chosen D-optimum designs provide efficient experimental schemes when the aim of the investigation is to obtain precise estimates of parameters. In the context of the current body of work, estimates of parameters refers to the enzyme kinetic parameters $V_{max}$ and $K_m$, but also the inhibition constant $K_i$. Traditionally, this experimental approach is performed on a grid of values of the design variables. But this approach may not be very efficient, in the sense that the parameter estimates ($V_{max}$, $K_m$ and $K_i$) have unnecessarily high variances. For good estimates of parameters the most efficient designs consist of clusters of replicates of a few sets of experimental conditions. The current study compares the application of such D-optimum designs with that of a conventional approach in assessing the competitive inhibitory potency of fluconazole and sertraline towards cytochrome P450s 2C9 and 2D6 respectively. In each case, the parameter estimates, namely $V_{max}$, $K_m$ and $K_i$, and were predicted well using the D-optimum design compared with those measured using the rich data sets, for both inhibitors. In conclusion, we have shown that D-optimality can provide more efficient designs for estimating the model parameters including, $K_i$. As such real cost savings can be made by the careful planning of studies using the theory of optimum experimental design.
Introduction

Cytochrome P450 enzymes play a major role in drug metabolism with CYPs 1A2, 2C9, 2C19, 2D6 and 3A4 being responsible for the majority of these reactions (Hasler et al., 1999). Altering the routes and/or rates of a metabolic reaction for a compound is particularly relevant with drugs that have a narrow therapeutic index, as small changes in the plasma concentration of the drug can potentially lead to an adverse effect. Such effects include reduction in efficacy or, even worse, toxicity. Inhibition of cytochrome P450s involved in the metabolic clearance of a drug, be it the drug itself and/or any co-administered drug, can potentially result in a metabolic drug-drug interaction (DDI). This is the most frequently observed DDI and the associated mechanism of action can be classed as being reversible, quasi-irreversible or irreversible. Of pertinence to the current work are reversible inhibitors whose mechanism of action can be further sub-divided as being competitive, non-competitive or uncompetitive (Houston et al., 2003). Mixed-inhibition has also been reported but is less common (Houston et al., 2003).

In a typical cytochrome P450 kinetic reaction, the enzyme binds substrate and metabolises it into associated products. The binding step is reversible while the catalytic step irreversible, and is written as the following chemical model

$$E + S \leftrightarrow ES \rightarrow E + P$$

where S, E and P denote substrate, enzyme and product, respectively. The reaction rate ($v$) is represented by the standard Michaelis-Menten model

$$v = \frac{V_{\text{max}}[S]}{K_m + [S]}$$

\[(1)\]
where $V_{\text{max}}$ denotes the maximum velocity of the enzyme, $[S]$ the concentration of the substrate and $K_m$ the Michaelis-Menten constant, which represents $[S]$ at which half of the maximum velocity is reached. The most prevalent mechanism of inhibition is competitive, where a compound binds reversibly to the enzyme preventing the binding of substrate and vice versa. The competition between substrate and inhibitor for the enzyme is represented by:

$$
EI 
\uparrow K_i 
\downarrow 
E + S 
\leftrightarrow ES \rightarrow E + P 
+ 
I
$$

where $K_i$ is the competitive inhibition constant. The initial rate of reaction $v$ follows the mechanistic model

$$v = \frac{V_{\text{max}}[S]}{K_m \left(1 + \frac{[I]}{K_i} + [S]\right)}, \quad (2)$$

where $[I]$ denotes the concentration of the inhibitor. For a fixed $[I]$, the limit of this function, when $[S]$ becomes infinitely large, is $V_{\text{max}}$. This means that the same maximum velocity is obtained irrespective of the concentration of the inhibitor. But, in its presence, higher concentrations of the substrate are needed to come equally close to the asymptote of $V_{\text{max}}$. Hence, the apparent $K_m$ increases with the concentration of the inhibitor, that is the reaction is slowed down.

The experimental procedure routinely used to determine these various parameters, involves incubating the enzyme source (e.g. human liver microsomes) with a specific cytochrome P450 substrate over a range of different concentrations. The drug whose potency (i.e. $K_i$) is being determined is also studied at various concentrations under
the aforementioned conditions, to see its effect on the amount of product being formed. From a pharmaceutical perspective, these P450 $K_i$ experiments are typically performed for compounds in the development phase and require the study be performed on multiple occasions. $K_i$ determinations are of course not limited to P450s, there are a host of other screens run within the pharmaceutical industry that generate $K_i$ values, for example target pharmacology potency screens.

Traditionally, this experimental paradigm is performed on a grid of values of the design variables. But this approach may not be very efficient and potentially generate superfluous data. Hence, application of D-optimum designs allows for good estimates of parameters, where the most efficient designs consist of clusters of replicates of a few sets of experimental conditions. For linear models the sets of conditions do not depend on the values of the unknown parameters in the model. The design situation is different for the model depicted by Equation 2, in which the parameters $K_m$ and $K_i$ enter nonlinearly. Then the location of the clusters of design points depends on the values of $K_m$ and $K_i$, although not on the value of $V_{max}$ which enters the model linearly.

The current study investigated if there was scope to optimize the traditional design (for estimating the inhibition constant $K_i$) through the application of D-optimum designs, and thus potentially impact efficiency by significantly reducing the sample numbers.
Methods

In Vitro Incubations.

All in vitro incubations were carried out using a MicroLab-STAR Autoload with 8 channels and a 96 Head (Hamilton Robotics, UK). The incubation mix consisted of (at their final concentrations): 50mM KH₂PO₄ buffer pH 7.4, 5mM MgCl₂, human liver microsomes (BD-Bioscience, Woburn, MA) and 1mM NADPH. Incubation times and protein concentration were selected such that they provided a linear reaction velocity with respect to product formation (as identified in preliminary experiments, data not shown). Incubation mixes were then supplemented with relevant inhibitors (fluconazole (Diflucan; CYP2C9) and sertraline (Zoloft; CYP2D6); Pfizer in-house chemical bank) at up to eight concentrations. Reactions were subsequently initiated by the addition of specific P450 probes (Pfizer in-house chemical bank): dextromethorphan (CYP2D6) or diclofenac (Voltaren; CYP2C9) across fifteen concentrations (0-50µM), at each inhibitor concentration (0-60µM). Reactions were terminated 1:2 in cold aceetonitrile (v/v) containing isotopically labelled dextrophan or 4-hydroxy diclofenac metabolites (50ng/mL). Terminated reaction mixtures were analyzed directly by HPLC-MS.

Samples were subsequently quantified using an Applied Biosystems/Sciex API 4000 QTRAP mass spectrometer in the positive ionization mode. A Phenomenex Synergi Fusion High pressure HPLC column, 2.0 mm × 20.0 mm, 2.5 µm particle size was used for chromatographic separation, at a flow rate 1 mL/min. A CTC auto sampler was used in conjunction with a Jasco XLC 3185PU high pressure, low dead volume, binary gradient pump, Jasco XLC 3067CO column oven and Jasco XLC 3080DG degasser (Youdim et al., 2008).
Data Analysis

Conventional data were analyzed by nonlinear regression analysis using Grafit 4 (Erithacus Software Ltd., Horley, Surrey, UK) applying models for Michaelis-Menten kinetics with inhibition (Equation 2). The criteria used to select and check the most appropriate model included visual inspection of the residuals, together with tests of independence of the errors and the constancy of error variance, and $F$ tests for the values of parameters.

Designing the Experiments

From a pharmaceutical perspective, P450 $K_i$ experiments are typically performed for compounds in the development phase and consist of taking measurements across a number of different concentration combinations of the substrate and inhibitor, with each combination normally repeated in triplicate. However, application of $K_i$ studies for compounds during discovery, where numbers will exceed that in development, necessitates a reduced design. As such, studies might be performed in singlicate. However, quite a different set-up is required if we want to optimize the experiment for parameter estimation. Then, the design usually consists of far fewer combinations of the concentrations, but each one replicated several times. We denote such designs by $\xi$ with a subscript $N$ to indicate the total number of observations to be taken, i.e.,

$$\xi_N = \left\{ x_1, \ldots, x_n \right\},$$

where $x_j$ denotes the design support points and $w_j = \frac{r_j}{N}$ represents the proportion of experimental effort at $x_j$, $j=1,\ldots,n$. Note that $\sum_{j=1}^n w_j = 1$ as $\sum_{j=1}^n r_j = N$. For
the current study, the support points \( x_j \) specify \( n \) combinations of substrate and inhibitor concentrations which come from the design region \( \Omega \), i.e., \( x_j = ([S], [I]) \) and \( \Omega = \{[0, [S]_{\text{max}}] \times [0, [I]_{\text{max}}] \} \), where \([S]_{\text{max}}\) and \([I]_{\text{max}}\) are the maximum allowable concentrations of the substrate and inhibitor, respectively. Although, in practice, setting such concentrations may be subject to error, in our experiments all \( x_j \) are determined with good precision, since the experiments were performed using an automated procedure.

In the design of the current experiments we assume that the errors in observing the rate of reaction \( v \) (Equation 2) are additive, independent, exhibit constant variance and, are approximately normally distributed. Analysis of conventional data supports these assumptions (data not shown). As such the appropriate method of parameter estimation is nonlinear least squares. If the model were linear the confidence region for the parameter estimates would be elliptical in shape, or ellipsoidal with three or more parameters. D-optimum designs choose the values of the \( x_j \) and \( r_j \) to minimize this volume. If the parameter estimates are uncorrelated this is equivalent to finding a design that minimizes the variances of the estimates. However, the estimates are usually not independent and D-optimality minimizes the generalized variance of the estimates defined as the determinant of the matrix of variances and covariances of the estimates. This determinant is proportional to the square root of the volume of the confidence ellipsoid. A succinct summary of the theory of optimum experimental design is given by Fedorov and Hackl (1997). For D-optimum design for nonlinear models see Atkinson et al., (2007).
Results and Discussion

The purpose of the current investigation is to compare a conventional approach with those that are D-optimum. Our conventional designs had \( n \) (the number of support points) equal to 120, consisting of a grid of 15 values of \([S]\) and eight of \([I]\) on a logarithmic scale, referred to as a “rich” data set. These rich data sets of substrate inhibitor pairings allowed estimation of the parameters for both sertraline against CYP2D6 and fluconazole against CYP2C9 (Table 1). In each case the inhibitory potencies obtained were consistent with those available in the literature for sertraline (Otton et al., 1993; Otton et al., 1996) and fluconazole (Youdim et al., 2008).

For many nonlinear models the D-optimum designs have to be found by numerical maximization. However, in our case, we were able to obtain the following analytical expressions for a D-optimum design for the competitive model which has the form:

\[
\xi_N = \left\{ \begin{array}{c}
([S]_{\text{max}},${}[I]_{\text{min}}) \\
\frac{1}{3} \\
\frac{1}{3} \\
\frac{1}{3} \end{array} \right\} 
\]

The model contains three parameters and, in this case the D-optimum design has an equal number of replicates at each of the three \(([S], [I])\) combinations. The values of the design variables \(s_2, s_3\) and \(i_3\) are:

\[
s_2 = \max\left\{ [S]_{\text{min}}, \frac{[S]_{\text{max}} K^0_m (K^0_i + [I]_{\text{min}})}{2 K^0_m K^0_i + 2 K^0_m [I]_{\text{min}} + [S]_{\text{max}} K^0_i} \right\} 
\]

\[
s_3 = \max\left\{ [S]_{\text{min}}, \min\left\{ \frac{K^0_m (K^0_i + [I]_{\text{max}})}{K^0_i}, [S]_{\text{max}} \right\} \right\} 
\]

\[
i_3 = \min\left\{ \frac{2 K^0_m [I]_{\text{min}} + [S]_{\text{max}} K^0_i + K^0_m K^0_i}{K^0_m}, [I]_{\text{max}} \right\} 
\]
where $K_m^0$ and $K_i^0$ denote the prior values of the model parameters. Note also that in our case $[I]_{\min} = 0$. The substrate and inhibitor pairs and their replications identified for each study (for the parameter priors estimated from the rich data sets) were:

for Diclofenac – fluconazole,

$$\xi_{30} = \begin{cases} 
(50, 0) & (4.6, 0) & (49.1, 60) \\
10 & 10 & 10 
\end{cases},$$

for Dextromethorphan – sertraline,

$$\xi_{21} = \begin{cases} 
(30, 0) & (3.4, 0) & (30, 20.2) \\
7 & 7 & 7 
\end{cases}.$$ 

In these displays the pairs such as (50, 0) indicate the values of [S] and [I] to be applied to ten or seven samples, depending on the reaction being studied. As can be seen, all design support points were on the border of the design region for at least one of the substrate and inhibitor concentrations and some were on the border for both concentrations. Given these D-optimum designs, D-efficiencies can be calculated for any other design (Atkinson et al., 2007). These efficiencies are such that a design with an efficiency of 50% requires twice as many trials as the D-optimum design to provide parameter estimates of the same accuracy. The diclofenac~fluconazole rich design had an efficiency of 0.254 on a per observation basis; the same efficiency for parameter estimation could be obtained with approximately 30 observations evenly split over the three support points of the D-optimum design as from the 120 observations from the rich design. For dextromethorphan~sertraline the D-efficiency of the rich design is even less, 0.182; a design with seven observations at each of the points of the D-optimum design provides greater efficiency than the rich design. The
difference in the number of observations between the two studies arises from the
dependence of the optimum design, and so of the efficiency of the design over a grid,
on the prior values of the parameters. By running experiments involving respectively
30 and 21 observations our theory predicts that we should obtain parameter estimates
that are as precise as those obtained from the 120 observations of the rich data sets.
The results of Table 1 show that the experimental analysis supports our theory.

The conventional assay approach was subsequently repeated using these three support
points with relevant replications of each. The estimates of the parameters determined
from this experimental approach, using MATLAB nonlinear least squares procedure
nlinfit, are shown in Table 1. The parameter estimates, namely $V_{\text{max}}$ and $K_m$, and were
predicted well using the D-optimum design compared with those measured using the
rich data sets. Of particular importance were the estimates of the inhibition constants.
For sertraline the $K_i$ towards CYP2D6 in the rich data set was estimated to be 2.6$nM$,
which agreed well with the value of 2.1$nM$ estimated using the D-optimum design.
The $K_i$ for fluconazole towards CYP2C9 also compared well with an estimated value
of 7.7$nM$ using the rich data set and 6.1$nM$ using the D-optimum design. More
important, for our demonstration of the cost saving implications of using D-optimum
designs, are the similarities between the standard errors for each parameter, given the
significant reduction in sample numbers compared with the rich-data approach.

The aforementioned D-optimum designs provide estimates of all three parameters
with small variances. However, estimating the substrate:inhibitor pairings and
relevant replicates requires retrospective analysis of the rich data set. Clearly there is
limited need within discovery, spending time establishing bespoke ‘optimized’
experimental designs to determine a $K_i$, when high-throughput approaches such as the IC$_{50}$ assay can provide some degree of guidance as to inhibitory potency (Jones et al., 2009). Depending on the mechanism, the $K_i$ may reflect half the IC$_{50}$ for competitive inhibitors or be equal to the IC$_{50}$ in the case of non-competitive inhibitors. However, where this approach may provide efficiency gains is for compounds progressing through the development pipeline and where regulatory agencies request more definitive measures of $K_i$. At this stage, there is likely to be sufficient information known about the parameter estimates such as the $K_m$ and $V_{max}$ for substrates (probes) in the actual liver microsome matrix against which the compound (inhibitor) is being tested against.

Given that $K_i$ estimates from IC$_{50}$ data could vary by 2-fold (dependant upon mechanism of inhibition) one could argue that there might be an advantage to having $K_i$ determined earlier during discovery to better guide predictions of drug-drug interactions Obach et al., 2005; Obach et al., 2006). However, such a strategy, where the conventional IC$_{50}$ assay would be replaced, must balance the need for data with cost effectiveness. The conventional approach for measuring $K_i$ during early discovery clearly goes against this doctrine as does the need to establish bespoke D-optimum designs for every compound. Hence, the next steps will be to establish optimum designs that are not governed by discrete point estimates of inhibitory potency (i.e IC$_{50}$), but rather designs that cover ‘regions’ of potency i.e. IC$_{50}$<1μM; 1-10μM, >10μM; a binning strategy often used by pharmaceutical companies as their first-tier approach to screen out compounds that pose a potential drug-drug interaction risk. Such approaches are currently being investigated at the authors’ institutions into this, using $D_3$-optimality which is an extension of D-optimality.
Here ‘s’ indicates that interest is in a subset of the parameters in the model, i.e. $K_i$. In the case of estimation of just a single parameter ($s = 1$), the design is found for which the estimate has minimum variance. These designs are rather different from the D-optimum designs shown above. In particular, the weights on the design points are often far from equal. In some cases the designs are even singular, putting weight, in our example, on less than three support points. While not immediately useful, since not all model parameters can be estimated, such designs provide a reference against which the $D_S$-efficiency of any other design can be calculated. Singular designs for nonlinear models have been reviewed previously (Atkinson et al., 2007). However, for illustrative purposes, the designs have been calculated for the current experimental data. Table 2 shows that the D-optimum design has a $D_S$-efficiency of 66.67% for detromethorphan-sertraline. This was found by comparison with the $D_S$-optimum design, which for numerical reasons, was constrained to have a weight of at least $1 \times 10^{-5}$ on the first support point. The rest of the weight is split equally between two points very close to those of the D-optimum design. The last line of the table shows the efficiencies for a design on these support points, with weights found to minimize the variance of the estimates of $K_i$. This design has a $D_S$-efficiency of 100% and a D-efficiency of only 4%. In between these extremes a series of designs are presented in which the weights are in the ratio $1: r: r$. The D-optimum design corresponds to $r = 1$. Designs for $r = 2$, 3 and 4 are also presented. As the weights become less equal the D-efficiency decreases slowly and that for $D_S$ increases. When $r = 4$ and the weights are $1/9$, $4/9$, $4/9$; the D and $D_S$ efficiencies are 83.99 and 88.89. The results for diclofenac-fluconazole are similar, where, as a result of $r$ increasing, the designs become less balanced, resulting in increased $D_S$-efficiency and decreased D-
efficiency. Values of 3 or 4 for \( r \) give designs that are not highly unbalanced and that have good efficiencies on both measures. It is not even necessary that \( r \) be an integer. For example, with 30 measurements the numbers at the three design points could be 4, 13 and 13, giving a value of 3.25 for \( r \) and a design with good D- and Ds-efficiencies. The optimum designs we have found depend both on the prior estimates of the parameters \( K_m \) and \( K_i \) (although not on \( V_{max} \)) and on the assumed model. In practice, these parameter values will not be as well known as they are in our examples. However, if the value of the IC\(_{50}\) is known, only one design parameter remains unknown. Optimum designs, and their efficiencies, can then be calculated for a series of parameter values and a design chosen with good efficiency over the range of values. If no such design can be found, D-optimality can be extended by using the prior distribution of the parameters as weights in the calculation of a ‘Bayesian’ design (Atkinson et al., 2007), which may require experiments at more than three combinations of concentrations. Likewise, compound optimality can be used (Atkinson et al., 2007) to find good designs when the mechanism of reaction is uncertain.

In conclusion, we have shown that D-optimality can provide more efficient designs for estimating model parameters, including inhibitory \( K_i \)'s. Such an approach may be of use for compounds that are further down the drug development pipeline, where prior knowledge of potency can be used to guide these mathematical designs. Having shown that D-optimal designs can be applied successfully, provides confidence to extend this approach to employ Ds-optimality, where there is less reliance of prior knowledge of parameter estimates.
Acknowledgements. The authors are grateful to Maurice Dickins and Barry Jones for their comments and guidance during this work.
References


Table 1. Parameter estimates and associated errors estimated using a rich data set, compared with those from a D-optimum design, for CYP2D6~ dextromethorphan & sertraline; CYP2C9~ diclofenac & fluconazole. Both rich designs contain 120 observations as opposed to 21 and 30 for the D-optimum designs.

<table>
<thead>
<tr>
<th>Sertaline~Dextromethorphan (CYP2D6)</th>
<th>Uniform (Rich Data Set)</th>
<th>D-optimum design</th>
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</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Estimate</td>
<td>Std Error</td>
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<tr>
<td>$V_{\text{max}}$ (nmol/min/mg)</td>
<td>0.73</td>
<td>0.01</td>
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<tr>
<td>$K_m$ ($\mu$M)</td>
<td>4.4</td>
<td>0.23</td>
</tr>
<tr>
<td>$K_i$ ($\mu$M)</td>
<td>2.6</td>
<td>0.14</td>
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<table>
<thead>
<tr>
<th>Fluconazole ~ Diclofenac (CYP2C9)</th>
<th>Uniform (Rich Data Set)</th>
<th>D-optimum design</th>
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</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Estimate</td>
<td>Std Error</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (nmol/min/mg)</td>
<td>1.8</td>
<td>0.03</td>
</tr>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>5.6</td>
<td>0.30</td>
</tr>
<tr>
<td>$K_i$ ($\mu$M)</td>
<td>7.7</td>
<td>0.55</td>
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Table 2 D- and D_s-optimum designs and their efficiencies. The support points are the three pairs of values of ([S], [I]) for each experiment.

<table>
<thead>
<tr>
<th>Support points</th>
<th>(30, 0)</th>
<th>(3.4, 0)</th>
<th>(30, 20.2)</th>
<th>Efficiency</th>
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<tr>
<td>Sertaline-Dextromethorphan (CYP2D6)</td>
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<tr>
<td>D-optimum design weights, r = 1</td>
<td>1/3</td>
<td>1/3</td>
<td>1/3</td>
<td>100</td>
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<tr>
<td>D_s on D</td>
<td>10^{-3}</td>
<td>≈ 1/2</td>
<td>≈ 1/2</td>
<td>4.07</td>
</tr>
<tr>
<td>D1-optimum design weights</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r = 2</td>
<td>1/5</td>
<td>2/5</td>
<td>2/5</td>
<td>95.24</td>
</tr>
<tr>
<td>r = 3</td>
<td>1/7</td>
<td>3/7</td>
<td>3/7</td>
<td>89.15</td>
</tr>
<tr>
<td>r = 4</td>
<td>1/9</td>
<td>4/9</td>
<td>4/9</td>
<td>83.99</td>
</tr>
<tr>
<td>Fluconazole ~ Diclofenac (CYP2C9)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-optimum design weights: r = 1</td>
<td>1/3</td>
<td>1/3</td>
<td>1/3</td>
<td>100</td>
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<td>D_s on D</td>
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<td>0.49</td>
<td>4.04</td>
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